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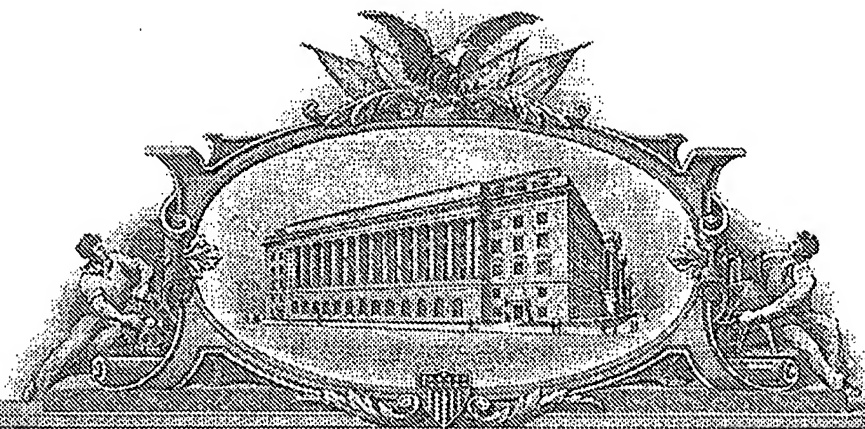
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## PROVISIONAL APPLICATION COVER SHEET

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INVENTOR (s) / APPLICANT (s)				
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)	
Trackman	Philip		1750 Central Avenue, Needham, MA 02492	
Palamakumbura	Amitha	H.	35 Vine Street, Apt # 206, Winchester, MA 01890	
Sonenshein	Gail	E.	549 Washington Street, Brookline, MA 02446	
Jeay	Sebastien		199 Winthrop Road, Apt. # 32, Brookline, MA 02445	
<input type="checkbox"/> Additional Inventors are being named on Page 2 attached.				
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Please recognize the following attorneys with powers in this application.

Stanley M. Schurgin, Reg. No. 20,979  
Charles L. Gagnebin III, Reg. No. 25,467  
Victor B. Lebovici, Reg. No. 30,864  
Beverly E. Hjorth, Reg. No. 32,033Holliday C. Heine, Reg. No. 34,346  
Gordon R. Moriarty, Reg. No. 38,973  
James F. Thompson, Reg. No. 36,699  
Richard E. Gamache, Reg. No. 39,196

Respectfully submitted,

SIGNATURE

Holliday C. Heine

DATE

Jan. 13, 2004TYPED or PRINTED NAME: Holliday C. Heine, Ph.D.REGISTRATION NO. 34,346

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## Use of the Pro-Peptide of Lysyl Oxidase as a Therapeutic Agent

Lysyl oxidase is an extracellular enzyme that is essential for the biosynthesis of normal cross-linked collagens and elastin. In addition, lysyl oxidase was shown to be a tumor suppressor, and is now known to be a "ras recision gene" (Contente et al., 1990; Kenyon et al., 1991), although the mechanism of this latter effect is unknown. Our new studies have investigated structure/function relationships that underlie the tumor suppressor activity of lysyl oxidase.

Lysyl oxidase is secreted as a 50 kDa inactive pro-enzyme, and it is then cleaved by procollagen C-proteinases extracellularly to generate the active 30 kDa enzyme and an 18- 20 kDa pro-peptide (Trackman et al., 1992; Panchenko et al., 1996; Uzel et al., 2001). Following experiments described herein, we developed the hypothesis that the released pro-peptide could be responsible for the ras-recision activity. Thus, we made the lysyl oxidase pro-peptide protein in *E. coli* by recombinant DNA technology, purified the pro-peptide, and confirmed its protein sequence. We next asked whether the pro-peptide influences the growth characteristics of c-H-ras transformed cells, first by cell cycle analyses and then by simple growth curves. We found that the pro-peptide both modulated the cell cycle and inhibited the growth of these cells. We then asked whether the pro-peptide inhibits the transformed phenotype by assessing its effect on the growth of ras-transformed cells in soft agar. Growth in soft agar is a hallmark of the transformed phenotype, and phenotypically normal cells are unable to grow and form colonies in soft agar. Results in replicate experiments show that the lysyl oxidase pro-peptide prevents the growth in soft agar of c-H-ras transformed cells. The mature active enzyme purified from bovine aorta was not able to inhibit growth in soft agar. Interestingly, cells transformed by a different oncogene (*c-myc*), which utilizes different signaling pathways, are not inhibited by the lysyl oxidase pro-peptide, demonstrating specificity and lack of toxicity of the pro-peptide. Furthermore, breast cancer cells transformed by a different oncogene *Her-2/neu*, which signals by a pathway that overlaps c-Ha-ras, was also inhibited by the lysyl oxidase pro-peptide in the growth-in-soft-agar assay.

The ability of pro-lysyl oxidase expression to inhibit ras-dependent transformation is due to its ability to inhibit PI3 kinase, Akt kinase and MEK kinase ultimately inhibiting NF- $\kappa$ B activation (Jeay et al., 2003). It appears that the lysyl oxidase pro-peptide directly inhibits activation of PI3, Akt and MEK kinases.

As the lysyl oxidase pro-peptide inhibits *ras*-dependent cell transformation, this pro-peptide, or active portions thereof, would be useful as anti-cancer therapeutic agents. Thus, the invention is directed to a therapeutic composition that includes an active portion of the lysyl oxidase pro-peptide in a pharmaceutically acceptable carrier substance and to methods of using such a therapeutic composition.

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Assays to detect the effectiveness of the lysyl oxidase pro-peptide in inhibiting, e.g., the growth of transformed cells can be used to determine active portions thereof. For example, using the soft agar assay described herein, or any cell culture assay, the activity of progressively smaller portions of the pro-peptide could be tested until the minimum sized active portion was determined.

Separate experiments carried out in normal differentiating osteoblast (bone cell) cultures show that the lysyl oxidase pro-peptide delays osteoblast differentiation, but interestingly appears to result ultimately in greater formation of a mineralized extracellular matrix. Thus, there may be therapeutic potential to treat osteopenia associated with diseases such as osteoporosis, or diabetic osteopenia, or other bone pathologies. Similarly, other disease conditions including kidney (Hendry and Sharpe, 2003), cardiovascular (Cvejic et al., 2000; Molkentin and Dorn, 2001), and immune system disorders (Cantrell, 2002; Schwartz, 2003; Wong et al., 2002), which occur via elevated ras-dependent signaling, seem likely to be improved by exposure to the lysyl oxidase pro-peptide.

This invention was made with Government Support under Contract Nos. DE 12425, CA 82742 and PO1-ES-11624-01 awarded by the NIH and Contract No. DAMD 17-03-1-0452 awarded by the Department of the Army. As such the Government has certain rights in this invention.

#### References

- Cantrell DA (2002) Transgenic analysis of thymocyte signal transduction. *Nat Rev Immunol* 2:20-27.
- Cvejic S, Jiang Y, Huang X. (2000) Signaling of G(alpha)(12) family of G proteins through a tyrosine kinase and a Ras-GAP. *Trends Cardiovasc Med.* 10:160-165.
- Contente S, Kenyon K, Rimoldi D, Friedman RM. (1990). Expression of gene *rrg* is associated with reversion of NIH 3T3 transformed by LTR-c-H-ras. *Science* 249:796-798.
- Hendry BM, Sharpe CC. (2003). Targeting Ras genes in kidney disease. *Nephron Exp Nephrol* 93:e129-33.
- Jeay S, Pianetti S, Kagan HM, Sonenshein GE. (2003). Lysyl oxidase inhibits ras-mediated transformation by preventing activation of NF-kappa B. *Mol Cell Biol* 23:2251-2263.
- Kenyon K, Contente S, Trackman PC, Tang J, Kagan HM, Friedman RM. (1991). Lysyl oxidase and *rrg* messenger RNA. *Science* 253:802.
- Molkentin JD, Dorn II GW 2<sup>nd</sup>. (2001) Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev Physiol.* 63:391-426.
- Panchenko MV, Stetler-Stevenson WG, Trubetskoy OV, Gacheru SN, Kagan HM. (1996). Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C- proteinase. *J Biol Chem* 271:7113-7119.
- Schwartz RH. (2003). T cell anergy. *Annu Rev Immunol* 21:305-34.
- Trackman PC, Bedell-Hogan D, Tang J, Kagan HM. (1992). Post-translational glycosylation and proteolytic processing of a lysyl oxidase precursor. *J Biol Chem* 267:8666-8671.
- Uzel MI, Scott IC, Babakhanlou-Chase H, Palamakumbura AH, Pappano WN, Hong HH et al. (2001). Multiple bone morphogenetic protein 1-related mammalian metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures. *J Biol Chem* 276:22537-22543.
- Wong CK, Zhang J, Ip WK, Lam CW. (2002). Intracellular signal transduction in eosinophils and its clinical significance. *Immunopharmacol Immunotoxicol* 24:165-86.

## The Pro-Peptide Domain of Lysyl Oxidase Mediates Its *Ras* Recision Activity<sup>1</sup>

Amitha H. Palamakumbura, Sébastien Jeay, Ying Guo, Nicole Pischon, Pascal Sommer, Gail E. Sonenshein, and Philip C. Trackman<sup>2</sup>

Division of Oral Biology, Boston University Goldman School of Dental Medicine (A.H.P, Y.G, N.P. and P.C.T.) and Department of Biochemistry, Boston University School of Medicine (S.J. and G.E.S.) Boston, MA 02118; and Institut de Biologie et Chimie des Protéines, CNRS UMR 5086, Université Claude Bernard, 69367 Lyon Cedex 07, France (P.S.)

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### Key words:

Lysyl oxidase, Ha-*ras*, transformation, BAPN, soft agar, suramin

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<sup>2</sup>To whom requests for reprints should be addressed, at Division of Oral Biology, Boston University Goldman School of Dental Medicine, 700 Albany St., Room W-210, Boston, MA 02118-2394. E-mail: trackman@bu.edu

<sup>3</sup>Abbreviations used are: *rrg*, ras recision gene; PI3K, phosphatidylinositol-3 kinase; BAPN,  $\beta$ -aminopropionitrile, FBS, fetal bovine serum; FACS, fluorescence activated cell sorting

## ABSTRACT

Lysyl oxidase is an extracellular enzyme critical for the normal biosynthesis of collagens and elastin. Lysyl oxidase was identified as a *ras* recision gene (*rrg*) with the ability to revert *ras*-mediated transformation, and its expression was shown to be down-regulated in human cancers. Since suramin inhibits growth factor signaling pathways and induces lysyl oxidase in *ras*-transformed NIH 3T3 cells, we sought to determine the effects of suramin on the phenotype of transformed cells. Suramin treatment resulted in a more normal phenotype as judged by growth rate, cell cycle parameters, and morphology.  $\beta$ -aminopropionitrile, the selective inhibitor of lysyl oxidase enzyme activity, was remarkably unable to block suramin-induced reversion, whereas ectopic anti-sense lysyl oxidase demonstrated that inhibition of lysyl oxidase gene expression was required for phenotypic reversion. Since lysyl oxidase is synthesized as a 50 kDa precursor and processed to a 30 kDa active enzyme and 18 kDa pro-peptide, the effects of these two products were then assessed individually. Here we report, for the first time, that the lysyl oxidase pro-peptide, and not the lysyl oxidase enzyme, has *rrg* activity, i.e., reduced the rate of proliferation and the ability of *ras*-transformed NIH 3T3 cells to grow in soft agar. Thus, the lysyl oxidase pro-peptide, which is released during extracellular proteolytic processing of pro-lysyl oxidase, functions to inhibit *ras*-dependent cell transformation.

## INTRODUCTION

Lysyl oxidase catalyzes oxidative deamination of peptidyl lysine and hydroxylysine residues in collagens, and peptidyl lysine residues in elastin. The resulting peptidyl aldehydes spontaneously condense and undergo oxidation reactions to form the lysine derived covalent cross-links required for the normal structural integrity of the extracellular matrix (1-3). Lysyl oxidase is synthesized as a 48 - 50 kDa pro-enzyme, secreted into the extracellular environment where it is then processed by proteolytic cleavage to a functional 30 kDa enzyme and an 18 kDa pro-peptide (4). Evidence supports that 30 kDa lysyl oxidase is active whereas the 50 kDa pro-enzyme is enzymatically inactive (5-7). Procollagen C-proteinases are active in processing pro-lysyl oxidase and are products of the *Bmp1* gene and the structurally related *Tll1* and *Tll2* genes (6-8).

The lysyl oxidase gene was found to inhibit the transforming activity of *ras* and was hence named the "*ras* recision gene" (*rrg*)<sup>3</sup> (9, 10). Lysyl oxidase is down-regulated in *ras*-transformed cells and in many cancer cell lines. Reduced lysyl oxidase levels are also observed in human cancers (9, 11-15), whereas in spontaneous revertants or upon induced phenotypic reversion higher normal levels of lysyl oxidase are again seen (9, 14). Conversely stable phenotypic revertants of *ras*-transfected NIH 3T3 cells return to a transformed phenotype upon transfection with an anti-sense lysyl oxidase vector (9, 10, 16). Moreover, anti-sense lysyl oxidase transfection triggers transformation of normal rat kidney fibroblasts (17). Thus, the lysyl oxidase gene has tumor suppressor activity. Recently, we showed that ectopic expression of lysyl oxidase in *ras*-transformed cells inhibits the activities of the phosphatidylinositol-3 kinase (PI3K), Akt and MEK kinases that lead to the activation of NF- $\kappa$ B (18).

Suramin is a polysulfonated naphthylurea, initially used in the treatment of trypanosomiasis and onchocerciasis (19). Suramin interrupts autocrine growth factor pathways by inhibiting the binding of growth factors to their receptors (19-23). We have recently shown that lysyl oxidase is dramatically up-regulated by suramin in c-Ha-*ras*-transformed RS485 fibroblasts due to its inhibition of an FGF-2 mediated autocrine pathway (24). The question is now raised whether suramin causes phenotypic reversion of RS485 cells, and whether this reversion depends on lysyl oxidase expression or activity. The results indicate that suramin-induced phenotypic reversion requires lysyl oxidase expression as expected, but reversion surprisingly does not require lysyl oxidase enzyme activity, and is mediated instead by the lysyl oxidase pro-peptide. These findings identify the 18 kDa lysyl oxidase pro-peptide as a novel inhibitor of *ras*-mediated transformation of fibroblasts.

## MATERIALS AND METHODS

*Chemicals.* Suramin was either kindly provided by the Division of Cancer Treatment Diagnosis and Centers, National Cancer Institute and Parke-Davis, or was purchased from Sigma (St Louis, MO). All other chemicals and reagents were purchased from Sigma or Gibco BRL (Rockville, MD).

*Cell culture.* RS485 cells are transformed by overexpression of c-Ha-ras in NIH 3T3 cells (25). PR4 cells are stable phenotypic revertants of RS485 cells obtained after treatment with  $\alpha/\beta$  interferon (26). Cells were plated onto 100 mm cell culture plates in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (FBS) plus 1% nonessential amino acids, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. Cultures were maintained at 37°C in a fully humidified atmosphere of 5% CO<sub>2</sub> in air. Cells in logarithmic growth phase, were dissociated with trypsin/EDTA, and inoculated at a desired density for each experiment.

*Growth curves.* To study cell growth rates, cells were plated in 6-well plates at a density of 35,000 cells/well and were grown in complete medium containing 10% FBS, as described above. Additions of suramin or  $\beta$ -aminopropionitrile (BAPN), when appropriate, were initiated 24 hours after plating. Media were changed every three days in the continuous presence of suramin or BAPN, as indicated for each experimental design in the Results section. Cell density was determined in triplicate every day by crystal violet staining, as described (27, 28). Cells were fixed with 10% formalin in PBS, washed with PBS, and then stained for 30 minutes with 0.1% crystal violet at room temperature with shaking. Unbound dye was then removed by washing with water until washes were colorless. Bound dye was then eluted with 10% acetic

acid, and quantitated by measuring the absorbance at 590 nm. For quantitative analyses of growth rates, the logarithmic value of absorbance vs time was plotted  $\pm$  standard deviation and the rates were calculated by linear regression analyses. In addition, data were plotted as total absorbance  $\pm$  SE vs time. Experiments were performed three times each with consistent findings.

*Lysyl oxidase enzyme activity.* PR4 cells were plated in 100 mm cell culture plates and then grown and re-fed every two days as described above in the constant presence of 0, 200 and 400  $\mu$ M BAPN for seven days until visually confluent. Cells were then re-fed with serum-free medium supplemented with 0.1% bovine serum albumin still in the constant presence or absence of BAPN. After 24 hours conditioned 0.3 ml aliquots of media samples were assayed in quadruplicate using a tritiated recombinant human tropoelastin as substrate as previously described (4). Incubations were performed at 37 °C for 90 minutes (29) and data were expressed as total cpm released  $\pm$  SE per culture.

*Cell cycle analysis.* RS485, NIH 3T3 and PR4 cells were plated on 100 mm plates and were grown until confluent with 0 or 150  $\mu$ M suramin. Cells ( $1.5 - 2 \times 10^6$ ) were then trypsinized, washed with PBS and fixed by washing with ice-cold 70% ethanol. Cells were stained with propidium iodide (50  $\mu$ g/ml) in PBS containing 2% FBS and was analyzed by flow cytometry using a FACScan flow cytometry with CELLQUEST acquisition and analysis software (Becton Dickinson Co, Bedford, MA).

*RNA isolation and Northern blot analysis.* Total RNA was isolated using the RNeasy – RNA isolation kit (Qiagen, Valencia, CA). Ten µg samples of denatured RNA were electrophoresed on a 1% agarose gel containing 18% formaldehyde. Gels were transferred in 10 x SSC by capillary blotting overnight to Gene Screen nylon membranes (Perkin Elmer Life Science, Boston, MA). The membranes were hybridized at 42°C as previously described (30) with labeled mouse lysyl oxidase probe (9, 10), prepared by random primer labeling (31). For normalization and as a measure of constant loading of gels, blots were stripped and rehybridized with a radiolabeled 18S rRNA probe (32). Autoradiograms were assessed and normalized by densitometric scanning on a Versa Doc Model 3000 Gel Documentation System and Quantity One Software (BioRad, Hercules, CA).

*Stable transfection of RS485 cells with an anti-sense lysyl oxidase expression vector.* Cells were grown in 100 mm cell culture dishes. At approximately 70% confluence, they were transfected with the anti-sense murine lysyl oxidase expression vector pCLO3 (17) (20 µg DNA/plate) using calcium phosphate precipitation method (33). As a control, RS485 cells were transfected with empty vector (pcDNA3). The transfected cells were selected using G418 (geneticin) at a final concentration of 400 µg/ml in the medium. Colonies were isolated from anti-sense lysyl oxidase and empty vector transfected RS485 cells using cloning cylinders (34) and cultured in 400 µg/ml G418. Cells were then plated onto 100 mm plates (250,000 cells/plate) without G418 and treated with 0 or 150 µM suramin. After 24 hours, cells were prepared for cell cycle and Northern analysis as described above.

*Lysyl oxidase pro-peptide coated cell culture plates.* Rat lysyl oxidase pro-peptide was expressed in *E. coli* and purified as described (35). The pro-peptide (200 – 400 µg/ml) was then dialyzed against 16 mM phosphate buffer, pH 7.8 for 5 hours and 6-well plates were coated with 0, 1, 5, or 10 µg pro-peptide in 1 ml water per well and left overnight under UV light in the cell culture hood to completely dry. RS485 cells were then plated at a density of 35,000 cells per well on the pro-peptide-coated plates and cultured until visual confluence. Cells were then prepared for cell cycle analysis as described above. To study the effect of pro-peptide on the growth rate of RS485 cells, 24-well plates were coated with 0, 0.2, 1, 2, or 4 µg pro-peptide in 350 µl water per well, as above, and the cells plated at a density of 7,000 cells/well. Cell density was determined in triplicate every day, by crystal violet staining as described above.

*Focus formation assay in soft agar:* c-Ha-ras-transformed NIH 3T3 cells and myc-transformed M158 cells were plated, in duplicate, at  $10^4$  cells/ml in top plugs consisting of complete Ham F-12 nutrient mixture medium and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine) in the presence of 2.5 µg purified bovine aorta lysyl oxidase enzyme (30 kDa form) (36), or with 2.5 µg recombinant rat lysyl oxidase pro-peptide (18 kDa form) (35), or the same volume of vehicle potassium phosphate (16 mM, pH 7.8). After 2 weeks incubation in a humidified incubator at 37°C, the colonies were stained with 0.5 ml of 0.0005% crystal violet and photographed using a digital camera coupled to a dissection microscope ( $\times 50$  magnification). Three random fields were counted from each duplicate samples, and average values presented  $\pm$  SD.

## RESULTS

*Effect of suramin on RS485 cell phenotype.* Treatment of c-Ha-ras-transformed NIH 3T3 cells (RS485 cell line) with suramin leads to the induction of lysyl oxidase (24). Here we investigated the effects of suramin on the rate of growth and morphology of RS485 cells, as an initial measure of cell phenotype. Cells were plated in 6-well plates and cultured for 24 hours and then grown in the continuous presence of 0, 100, 125, or 150  $\mu$ M suramin. As control, the growth of phenotypically normal NIH 3T3 cells in the absence of suramin was analyzed at the same time. Cell growth was determined by daily crystal violet staining of replicate wells (Figure 1). RS485 cells grew more rapidly than NIH 3T3 cells, as expected (25). Suramin significantly decreased the growth rate in a dose-dependent manner. Data show that 100, 125 and 150  $\mu$ M suramin decreased the growth rate by 38%, 49%, and 56%, respectively, calculated from linear regression analyses of log of absorbance vs. time. The growth rate of RS485 cells treated with 150  $\mu$ M suramin was similar to that of NIH 3T3 cells. Furthermore, treatment with suramin caused a dose-dependent change in the morphology of RS485 cells with cells appearing less transformed, i.e., flatter and contact inhibited in the presence of 150  $\mu$ M suramin (inset, Figure 1 and data not shown).

We next determined the effects of suramin on cell cycle progression of RS485 cell cultures. As shown in Table I, fluorescence activated cell sorting (FACS) indicated that treatment with 100 or 150  $\mu$ M suramin increased the percentage of RS485 cells in G<sub>1</sub> and decreased S phase in dose-dependent manner. The proportion of cells in G<sub>1</sub> and S phase in cultures treated with 150  $\mu$ M suramin is nearly identical to untreated phenotypically normal NIH 3T3 cells (Table I). Thus, treatment with 150  $\mu$ M suramin changed the morphology, cell cycle, and growth rate of RS485 cells resulting in a more normal phenotype.

*BAPN-mediated inhibition of lysyl oxidase enzyme activity fails to prevent phenotypic reversion induced by suramin.* It is generally assumed, although never directly tested, that lysyl oxidase dependent phenotypic reversion and tumor suppressor activity depends on its enzyme activity. To directly test the role of lysyl oxidase enzyme activity, we used BAPN (37), a selective inhibitor of lysyl oxidase enzyme activity, on the phenotypic changes in RS485 cells following treatment with 150  $\mu$ M suramin, which induces lysyl oxidase activity by about 2.5-fold (24). RS485 cell growth was assessed in the absence or presence of either 150  $\mu$ M suramin or 400  $\mu$ M BAPN, or a combination of both 150  $\mu$ M suramin and 400  $\mu$ M BAPN. This concentration of BAPN effectively inhibits lysyl oxidase in organ and cell cultures (38). As shown in Figure 2A, suramin decreased the growth rate of RS485 cells whereas BAPN had no effect on the growth rate. Surprisingly, BAPN did not reverse or affect in any detectable way the suramin-mediated inhibition of RS485 cell growth.

Studies performed with the stable phenotypic revertant cell line PR4 demonstrated that lysyl oxidase expression specifically is required to maintain the normal phenotype (9, 10). These studies utilized anti-sense transfection methodology to reduce lysyl oxidase expression resulting in transformation, but did not directly investigate the role of lysyl oxidase enzyme activity in phenotypic reversion. If lysyl oxidase enzyme activity were not required for the normal phenotype of PR4 cells, then BAPN would fail to cause re-transformation. Growth curves were generated for PR4 cells in the presence of 0 or 400  $\mu$ M BAPN. As shown in Figure 2B, BAPN did not affect the growth rate of PR4 cells. Furthermore, BAPN did not change the morphology of PR4 cells (data not shown). Assays of PR4 cell culture media confirmed that cells grown without BAPN produce lysyl oxidase enzyme activity (14,000  $\pm$  400 dpm/  $\times 10^6$  cells), whereas no lysyl oxidase enzyme activity was detected in the medium of cultures grown in the continuous

presence of both 200  $\mu$ M and 400  $\mu$ M BAPN using a highly sensitive assay for lysyl oxidase enzyme activity (4). Taken together, these data show that growth inhibition of RS485 cells by suramin does not depend on lysyl oxidase enzyme activity. Similarly, inhibition of lysyl oxidase enzyme activity does not affect growth of stable phenotypic revertants that require lysyl oxidase expression for the normal cell phenotype.

*Inhibition of lysyl oxidase expression reduces phenotypic reversion induced by suramin.*

Data presented above indicate that suramin causes phenotypic changes in RS485 cells, and that lysyl oxidase enzyme activity does not contribute to this effect. To verify that the suramin-induced phenotypic changes depend upon lysyl oxidase expression, we generated stable anti-sense lysyl oxidase transfected RS485 cell clones. Nine anti-sense lysyl oxidase transfected clones and six empty vector transfected clones were generated and grown in the presence and absence of 150  $\mu$ M suramin, and after 24 hours cells were fixed and subjected to cell cycle analysis. Non-transfected RS485 cells were analyzed as an additional control. Figure 3 shows the differences in the percentage of cells in G1 and S phase as a function of suramin treatment. In empty vector transfected clones, suramin increased the average number of cells in G1 by 20.8%, and decreased the average number of cells in S phase by 13.5% (bar N in Figure 3). As expected, these values are not significantly different from non-transfected cells (Figure 3, compare V to N). In contrast, anti-sense lysyl oxidase transfected clones show only a 10.6% increase in the percentage of cells in G1 phase and 6.9% decrease in the S phase after suramin treatment. These changes are significantly smaller than either of the control groups of cells (Figure 3, compare L; to V or N,  $p < 0.01$ ).

To confirm that lysyl oxidase expression is actually diminished by anti-sense lysyl oxidase transfection, the ability of 150  $\mu$ M suramin to induce the low steady state mRNA levels of lysyl oxidase in clones was assessed after 24 hours of treatment by Northern blot analysis with normalization to 18S rRNA signals. Suramin treatment led to an average 8-fold increase in lysyl oxidase mRNA levels in the six empty vector-transfected clones assayed, consistent with previous studies on non-transfected RS485 cells (24). In contrast, an average 1.8-fold increase in lysyl oxidase mRNA levels occurred in the nine anti-sense lysyl oxidase transfected clones, with only one clone increasing by more than 2-fold. A Northern blot of RNA from representative empty vector and anti-sense transfected clones is shown in Figure 4. Thus, the induction of steady state lysyl oxidase mRNA levels by suramin is inhibited by the anti-sense lysyl oxidase transfection, as expected. Taken together, these data demonstrate that anti-sense lysyl oxidase transfected cells have significantly diminished suramin-induced cell cycle changes compared to those of empty-vector transfected or non-transfected RS485 cells. These data support the notion that lysyl oxidase expression, but not enzyme activity, plays a role in mediating the phenotypic effects of suramin on RS485 cells.

*Lysyl oxidase pro-peptide causes phenotypic reversion of RS485 cells.* The biosynthesis of lysyl oxidase includes extracellular proteolysis of 48 - 50 kDa pro-lysyl oxidase by procollagen C-proteinases to release the 30 kDa lysyl oxidase enzyme and an 18 kDa cationic pro-peptide. The question, therefore, arises as to whether the released pro-peptide contributes to phenotypic reversion. To determine the effect of recombinant rat lysyl oxidase pro-peptide on the phenotype, RS485 cells were plated on 6-well plates that had been coated with 0, 1, 5, or 10  $\mu$ g pro-peptide per well. This experimental approach was taken due to the poor solubility of the

pro-peptide in cell culture media and physiologic buffers. After 4 days, cells ( $1-2 \times 10^6$ ) were harvested and subjected to cell cycle analysis (Table II). The percentage of cells in G1 phase increased in the presence of the pro-peptide from 53.5% to 61.1%, while the percentage of cells in S phase decreased from 22.6% to 14.9%, suggesting that the lysyl oxidase pro-peptide has a role in altering the cell cycle of RS485 cells.

We next investigated the effects of the lysyl oxidase pro-peptide on the growth of RS485 cells by culturing on pro-peptide coated 24-well plates. As shown in Figure 5, pro-peptide decreased the growth of RS485 cells in a dose-dependent manner. Linear regression analyses of plots of the log of absorbance vs time demonstrated dose-dependent growth inhibition of 4.1%, 5.6%, 8.9%, and 11.9% with 0.2, 1, 2 or 4  $\mu$ g of lysyl oxidase pro-peptide, respectively. Taken together, the data suggest that the influence of the lysyl oxidase pro-peptide on growth and on cell cycle progression of these cells contributes to phenotypic reversion.

*Lysyl oxidase pro-peptide, and not the active enzyme, inhibits growth of c-Ha-ras transformed cells in soft agar.* A hallmark of transformed cells is the ability to grow in soft agar and to form colonies, whereas non-transformed cells are unable to grow when suspended in soft agar. The respective effects of active 30 kDa lysyl oxidase enzyme, and of the 18 kDa lysyl oxidase pro-peptide vs vehicle control on the ability of *ras*-transformed NIH 3T3 cells to grow in soft agar were determined. Lysyl oxidase pro-peptide was strongly inhibitory, whereas the 30 kDa lysyl oxidase enzyme was unable to inhibit growth of *ras*-transformed cells in soft agar (Figure 6). In two separate experiments an average 80% reduction in colony formation was observed. To verify the specificity of the growth inhibitory effects of the pro-peptide on *c-myc*-transformed M158 fibroblasts was determined, given that lysyl oxidase mediated reversion

appears to be selective for *ras*-transformed cells (9, 10). Neither lysyl oxidase pro-peptide nor the 30 kDa lysyl oxidase enzyme inhibited the growth of *c-myc* transformed M185 fibroblasts. Thus, the 18 kDa lysyl oxidase pro-peptide, and not the active lysyl oxidase enzyme, inhibits *ras*-dependent transformation.

## DISCUSSION

This report shows for the first time that the *ras*-recision activity of lysyl oxidase depends substantially on the pro-peptide domain, and not on lysyl oxidase enzyme activity. Since diminished lysyl oxidase expression in some way contributes to the transformed phenotype, it has generally been assumed that lysyl oxidase enzyme activity is related to the tumor suppressor activity of lysyl oxidase, and, therefore, that diminished lysyl oxidase activity promotes the transformed phenotype. However, BAPN, the specific inhibitor of lysyl oxidase enzyme activity, did not prevent suramin-mediated reversion of the transformed phenotype, which is accompanied by increased lysyl oxidase expression. These findings were confirmed in the stable phenotypic revertant cell line PR4, that requires lysyl oxidase expression for normal phenotype maintenance; yet inhibition of lysyl oxidase activity with BAPN failed to re-transform these cells. Similarly, BAPN failed to block the ability of ectopic lysyl oxidase expression to prevent growth of *ras*-transformed fibroblasts in soft agar (data not shown). The lack of effect of BAPN on lysyl oxidase-dependent phenotype control is interesting. Intracellular localization of mature lysyl oxidase has been shown to occur via normal extracellular processing of pro-lysyl oxidase, followed by uptake of mature lysyl oxidase (39). Given that BAPN is an irreversible inhibitor of lysyl oxidase (37), it follows that both extracellular and intracellular lysyl oxidase activity are susceptible to inhibition by BAPN. These findings, therefore suggest that neither extracellular nor intracellular lysyl oxidase activity contribute significantly to inhibiting the transformed cell phenotype.

The importance of lysyl oxidase expression in maintaining a normal cell phenotype in suramin treated RS485 cells was supported by anti-sense transfection studies. Separate consistent studies demonstrate that 150  $\mu$ M suramin substantially inhibits colony formation in soft agar of

*ras*-transformed cells, but not *c-myc* transformed cells (data not shown). Most important, using recombinant lysyl oxidase pro-peptide, we demonstrated that the lysyl oxidase pro-peptide itself directly stimulates phenotypic reversion of *ras*-transformed cells, as judged by rate of proliferation, cell cycle and colony formation in soft agar. This contrasts with the absence of an effect of active lysyl oxidase on colony formation of *ras*-transformed cells in soft agar. These studies identify an activity of the lysyl oxidase pro-peptide that may ultimately prove to be of therapeutic significance in the treatment of cancers in which *ras*-dependent pathways are abnormally active.

A concept that has gained increasing experimental support is that many proteins have multiple biological functions (40). A recently reported example is the “house-keeping” cytoplasmic enzyme GAPDH, which has been unexpectedly found in the nucleus as a component of a transcription complex (41). It is unknown whether GAPDH enzyme activity plays a role in its activity as a transcription complex component, though its co-activator activity does depend on NAD<sup>+</sup> binding. Lens crystallins are structural proteins found in the lens that in other contexts serve as enzymes (42). Similarly, there is an increasing appreciation for biological activities of pro-peptides of structural proteins that are released as a result of biosynthetic proteolytic processing and maturation. C-pro-peptides of type I and type II collagen are ligands for  $\alpha 2\beta 1$  integrins and they inhibit collagen gene transcription (43-46). The C-pro-peptide of type I collagen promotes attachment of osteoblasts, and is chemotactic for endothelial cells (47, 48). A variant of the amino terminal pro-peptides of type II collagen binds and modulates the activity of TGF- $\beta 1$  and BMP-2 in developing cartilage (49). Endostatin is a 20 kDa cationic protein derived from C-terminal extensions of type XVIII procollagen that inhibits angiogenesis (50), an activity that is receiving much attention as a therapeutic approach to treat cancer. The finding

reported here that the lysyl oxidase pro-peptide has biological function as an inhibitor of cell transformation provides a new and important example of a distinct biological activity derived from an extracellular protein precursor.

Structural features of the lysyl oxidase pro-peptide are interesting. The biosynthesis of lysyl oxidase occurs by secretion of a 50 kDa precursor, followed by extracellular proteolytic processing to form active 30 kDa lysyl oxidase and the 18 – 20 kDa pro-peptide (5-7). Unlike the anionic C-terminal region of pro-lysyl oxidase that becomes the active enzyme after processing (6, 7), the N-terminal pro-peptide region is rich in arginine and is cationic with a calculated pI of 12.5 for the mouse and rat proteins. We hypothesize that the highly basic character of the lysyl oxidase pro-peptide could facilitate its uptake by cells where it might exert its biological function, possibly entering cells even in the absence of a specific receptor. Cell membranes are permeable to arginine-rich basic proteins, and uptake of these basic proteins is mediated by heparin sulfate proteoglycans (51). The arginine-rich highly basic pro-peptide region of lysyl oxidase is less well conserved between species than the mature enzyme (52), but it contains two blocks of 33 and 37 amino acids residues in length, respectively, that are nearly perfectly conserved between mouse and human, and highly conserved in chicken lysyl oxidase. These regions are residues 26 – 59, and 77 – 114, respectively, in the mouse lysyl oxidase sequence. This high homology suggests that biological activities of the lysyl oxidase pro-peptide reside in these conserved sequences. Lysyl oxidase is a member of a multi-gene family, and it is notable that the sequence of the lysyl oxidase pro-peptide region is not well conserved among other lysyl oxidase family members, whereas the catalytic domains are well conserved. We have recently shown that lysyl oxidase is dramatically up-regulated by suramin in c-Ha-*ras*-transformed fibroblasts due to its inhibition of an FGF-2 mediated autocrine pathway (24). Lysyl

oxidase itself, and not the lysyl oxidase like genes, has been consistently identified in screens for tumor suppressors and is expressed at low levels in transformed cells and at higher levels in phenotypically normal cells (9, 12). The finding of phenotype modulating activities occurring in regions of lysyl oxidase that are located in the unique pro-peptide domain may help to explain why lysyl oxidase itself is a tumor suppressor. Comparisons of the lysyl oxidase pro-peptide sequence with data bases have so far not revealed clues regarding the mechanisms by which the lysyl oxidase pro-peptide functions to inhibit cell transformation. Future studies will investigate the structure/function relationships and mechanism of action of the lysyl oxidase pro-peptide in cell phenotype control.

## REFERENCES

1. Kagan, H. M. Characterization and regulation of lysyl oxidase. *In*: R. P. Mecham (ed.), *Biology and Regulation of Extracellular Matrix: A Series. Regulation of Matrix Accumulation.*, Vol. 1, pp. 321-398: Academic Press, Orlando, 1986.
2. Kagan, H. M. and Trackman, P. C. Properties and function of lysyl oxidase. *Am J Respir Cell Mol Biol*, 5: 206-210, 1991.
3. Kagan, H. M. and Li, W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem*, 88: 660-672, 2003.
4. Bedell-Hogan, D., Trackman, P., Abrams, W., Rosenbloom, J., and Kagan, H. Oxidation, cross-linking, and insolubilization of recombinant tropoelastin by purified lysyl oxidase. *J Biol Chem*, 268: 10345-10350, 1993.
5. Trackman, P. C., Bedell-Hogan, D., Tang, J., and Kagan, H. M. Post-translational glycosylation and proteolytic processing of a lysyl oxidase precursor. *J Biol Chem*, 267: 8666-8671., 1992.
6. Panchenko, M. V., Stetler-Stevenson, W. G., Trubetskoy, O. V., Gacheru, S. N., and Kagan, H. M. Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C- proteinase. *J Biol Chem*, 271: 7113-7119., 1996.
7. Uzel, M. I., Scott, I. C., Babakhanlou-Chase, H., Palamakumbura, A. H., Pappano, W. N., Hong, H. H., Greenspan, D. S., and Trackman, P. C. Multiple bone morphogenetic protein 1-related mammalian metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures. *J Biol Chem*, 276: 22537-22543., 2001.

8. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. Bone morphogenetic protein-1: the type I procollagen C-proteinase. *Science*, 271: 360-362, 1996.
9. Contente, S., Kenyon, K., Rimoldi, D., and Friedman, R. M. Expression of gene *rrg* is associated with reversion of NIH 3T3 transformed by LTR-c-H-ras. *Science*, 249: 796-798., 1990.
10. Kenyon, K., Contente, S., Trackman, P. C., Tang, J., Kagan, H. M., and Friedman, R. M. Lysyl oxidase and *rrg* messenger RNA. *Science*, 253: 802., 1991.
11. Kuivaniemi, H., Korhonen, R. M., Vaheri, A., and Kivirikko, K. I. Deficient production of lysyl oxidase in cultures of malignantly transformed human cells. *FEBS Lett*, 195: 261-264, 1986.
12. Ren, C., Yang, G., Timme, T. L., Wheeler, T. M., and Thompson, T. C. Reduced lysyl oxidase messenger RNA levels in experimental and human prostate cancer. *Cancer Res*, 58: 1285-1290., 1998.
13. Krzyzosiak, W. J., Shindo-Okada, N., Teshima, H., Nakajima, K., and Nishimura, S. Isolation of genes specifically expressed in flat revertant cells derived from activated ras-transformed NIH 3T3 cells by treatment with azatyrosine. *Proc Natl Acad Sci U S A*, 89: 4879-4883., 1992.
14. Hajnal, A., Klemenz, R., and Schafer, R. Up-regulation of lysyl oxidase in spontaneous revertants of H-ras- transformed rat fibroblasts. *Cancer Res*, 53: 4670-4675., 1993.
15. Hamalainen, E. R., Kemppainen, R., Kuivaniemi, H., Tromp, G., Vaheri, A., Pihlajaniemi, T., and Kivirikko, K. I. Quantitative polymerase chain reaction of lysyl oxidase mRNA in malignantly transformed human cell lines demonstrates that their low

lysyl oxidase activity is due to low quantities of its mRNA and low levels of transcription of the respective gene. *J Biol Chem*, 270: 21590-21593., 1995.

16. Trackman, P. C., Pratt, A. M., Wolanski, A., Tang, S. S., Offner, G. D., Troxler, R. F., and Kagan, H. M. Cloning of rat aorta lysyl oxidase cDNA: complete codons and predicted amino acid sequence. *Biochemistry*, 29: 4863-4870., 1990.
17. Giampuzzi, M., Botti, G., Cilli, M., Gusmano, R., Borel, A., Sommer, P., and Di Donato, A. Down-regulation of lysyl oxidase-induced tumorigenic transformation in NRK-49F cells characterized by constitutive activation of ras proto- oncogene. *J Biol Chem*, 276: 29226-29232., 2001.
18. Jeay, S., Pianetti, S., Kagan, H. M., and Sonenshein, G. E. Lysyl oxidase inhibits ras-mediated transformation by preventing activation of NF-kappa B. *Mol Cell Biol*, 23: 2251-2263., 2003.
19. Sullivan, K. A., Kim, B., Buzdon, M., and Feldman, E. L. Suramin disrupts insulin-like growth factor-II (IGF-II) mediated autocrine growth in human SH-SY5Y neuroblastoma cells. *Brain Res*, 744: 199-206., 1997.
20. Zanghi, J. A., Renner, W. A., Bailey, J. E., and Fussenegger, M. The growth factor inhibitor suramin reduces apoptosis and cell aggregation in protein-free CHO cell batch cultures. *Biotechnol Prog*, 16: 319-325., 2000.
21. Fukumoto, M., Takahashi, J. A., Murai, N., Ueba, T., Kono, K., and Nakatsu, S. Induction of apoptosis in glioma cells: an approach to control tumor growth by blocking basic fibroblast growth factor autocrine loop. *Anticancer Res*, 20: 4059-4065., 2000.

22. Mietz, H., Chevez-Barrios, P., Feldman, R. M., and Lieberman, M. W. Suramin inhibits wound healing following filtering procedures for glaucoma. *Br J Ophthalmol*, 82: 816-820., 1998.
23. Coffey, R. J., Jr., Leof, E. B., Shipley, G. D., and Moses, H. L. Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. *J Cell Physiol*, 132: 143-148., 1987.
24. Palamakumbura, A. H., Sommer, P., and Trackman, P. C. Autocrine growth factor regulation of lysyl oxidase expression in transformed fibroblasts. *J Biol Chem*, 278: 30781-30787, 2003.
25. Chang, E. H., Furth, M. E., Scolnick, E. M., and Lowy, D. R. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature*, 297: 479-483, 1982.
26. Samid, D., Flessate, D. M., and Friedman, R. M. Interferon-induced revertants of ras-transformed cells: resistance to transformation by specific oncogenes and retransformation by 5-azacytidine. *Mol Cell Biol*, 7: 2196-2200, 1987.
27. Kueng, W., Silber, E., and Eppenberger, U. Quantification of cells cultured on 96-well plates. *Anal Biochem*, 182: 16-19., 1989.
28. Gillies, R. J., Didier, N., and Denton, M. Determination of cell number in monolayer cultures. *Anal Biochem*, 159: 109-113., 1986.
29. Hong, H. H., Uzel, M. I., Duan, C., Sheff, M. C., and Trackman, P. C. Regulation of lysyl oxidase, collagen, and connective tissue growth factor by TGF- $\beta$ 1 and detection in human gingiva. *Lab Invest*, 79: 1655-1667, 1999.

30. Feres-Filho, E. J., Choi, Y. J., Han, X., Takala, T. E., and Trackman, P. C. Pre- and post-translational regulation of lysyl oxidase by transforming growth factor- $\beta$  1 in osteoblastic MC3T3-E1 cells. *J Biol Chem*, 270: 30797-30803., 1995.
31. Feinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem*, 132: 6-13., 1983.
32. Hillis, D. M. and Dixon, M. T. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol*, 66: 411-453., 1991.
33. Graham, F. L. and van der Eb, A. J. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, 52: 456-467., 1973.
34. Southern, P. J. and Berg, P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet*, 1: 327-341, 1982.
35. Hong, H. H., Pischon, N., Santana, R. B., Palamakumbura, A. H., Babakhanlou-Chase, H., Gantz, D., Guo, Y., Uzel, M. I., Ma, D., and Trackman, P. C. A role for lysyl oxidase regulation in the control of collagen deposition in differentiating osteoblast cultures. *J. Cellular Physiol.*, *in press*, 2004.
36. Palamakumbura, A. H. and Trackman, P. C. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem*, 300: 245-251., 2002.
37. Tang, S. S., Trackman, P. C., and Kagan, H. M. Reaction of aortic lysyl oxidase with  $\beta$ -aminopropionitrile. *J Biol Chem*, 258: 4331-4338., 1983.
38. Kagan, H. M. and Sullivan, K. A. Lysyl oxidase: preparation and role in elastin biosynthesis. *Methods Enzymol*, 82 Pt A: 637-650, 1982.

39. Nellaiappan, K., Risitano, A., Liu, G., Nicklas, G., and Kagan, H. M. Fully processed lysyl oxidase catalyst translocates from the extracellular space into nuclei of aortic smooth-muscle cells. *J Cell Biochem*, 79: 576-582, 2000.
40. McKnight, S. Gene switching by metabolic enzymes--how did you get on the invitation list? *Cell*, 114: 150-152, 2003.
41. Zheng, L., Roeder, R. G., and Luo, Y. S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell*, 114: 255-266, 2003.
42. Wistow, G. and Piatigorsky, J. Recruitment of enzymes as lens structural proteins. *Science*, 236: 1554-1556, 1987.
43. Wu, C. H., Donovan, C. B., and Wu, G. Y. Evidence for pretranslational regulation of collagen synthesis by procollagen propeptides. *J Biol Chem*, 261: 10482-10484, 1986.
44. Aycock, R. S., Raghow, R., Stricklin, G. P., Seyer, J. M., and Kang, A. H. Post-transcriptional inhibition of collagen and fibronectin synthesis by a synthetic homolog of a portion of the carboxyl-terminal propeptide of human type I collagen. *J Biol Chem*, 261: 14355-14360, 1986.
45. Davies, D., Tuckwell, D. S., Calderwood, D. A., Weston, S. A., Takigawa, M., and Humphries, M. J. Molecular characterisation of integrin-procollagen C-propeptide interactions. *Eur J Biochem*, 246: 274-282, 1997.
46. Mizuno, M., Fujisawa, R., and Kuboki, Y. The effect of carboxyl-terminal propeptide of type I collagen (c-propeptide) on collagen synthesis of preosteoblasts and osteoblasts. *Calcif Tissue Int*, 67: 391-399, 2000.

47. Mizuno, M., Kitafima, T., Tomita, M., and Kuboki, Y. The osteoblastic MC3T3-E1 cells synthesized C-terminal propeptide of type I collagen, which promoted cell-attachment of osteoblasts. *Biochim Biophys Acta*, 1310: 97-102, 1996.
48. Palmieri, D., Camardella, L., Ulivi, V., Guasco, G., and Manduca, P. Trimer carboxyl propeptide of collagen I produced by mature osteoblasts is chemotactic for endothelial cells. *J Biol Chem*, 275: 32658-32663, 2000.
49. Zhu, Y., Oganessian, A., Keene, D. R., and Sandell, L. J. Type IIA procollagen containing the cysteine-rich amino propeptide is deposited in the extracellular matrix of prechondrogenic tissue and binds to TGF- $\beta$ 1 and BMP-2. *J Cell Biol*, 144: 1069-1080, 1999.
50. Marnaros, A. G. and Olsen, B. R. The role of collagen-derived proteolytic fragments in angiogenesis. *Matrix Biol*, 20: 337-345, 2001.
51. Belting, M. Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends Biochem Sci*, 28: 145-151, 2003.
52. Mariani, T. J., Trackman, P. C., Kagan, H. M., Eddy, R. L., Shows, T. B., Boyd, C. D., and Deak, S. B. The complete derived amino acid sequence of human lysyl oxidase and assignment of the gene to chromosome 5 (extensive sequence homology with the murine ras recision gene). *Matrix*, 12: 242-248, 1992.

Table I

Effects of Suramin on the Cell Cycle Progression of RS485 Cells

Cell Type	G1%	S%	G2, M%
RS485	43.3 ± 3.6	30.8 ± 4.6	25.8 ± 1.1
RS485 with 100 $\mu$ M suramin	59.4 ± 1.3	21.5 ± 2.4	19.1 ± 7.9
RS485 with 150 $\mu$ M suramin	67.4 ± 0.2	19.0 ± 1.7	13.6 ± 1.9
NIH 3T3	66.2 ± 2.1	17.4 ± 0.4	16.4 ± 3.0

RS485 cells were grown in the presence of 0, 100, or 150  $\mu$ M suramin for 2 days. Alternatively, NIH 3T3 cells were grown in the absence of suramin, as control. Cells ( $1.5 - 2 \times 10^6$ ) were fixed with 70% ethanol and stained with propidium iodide and analyzed by flow cytometry. Data shown are the averages  $\pm$  SD of experiments performed three times.

Table II  
Effects of the Lysyl Oxidase Pro-peptide on Cell Cycle Progression of RS485 Cells

Cell Type	G1%	S%	G2, M%
RS485	53.5 ± 0.7	22.6 ± 0.8	23.8 ± 1.6
RS485 with 1 µg pro-peptide	57.3 ± 1.7	20.8 ± 2.3	21.2 ± 1.9
RS485 with 5 µg pro-peptide	59.2 ± 2.3	19.1 ± 1.6	21.7 ± 2.1
RS485 with 10 µg pro-peptide	61.1 ± 2.4	14.9 ± 0.5	23.9 ± 1.9

RS485 cells were grown on 0, 1, 5, or 10 µg of propeptide coated 6-well plates and cultured for 2 days. Cells ( $1.5 - 2 \times 10^6$ ) were fixed with 70% ethanol and stained with propidium iodide and analyzed by flow cytometry. Data shown are the averages ± SD of experiments performed three times.

## FIGURE LEGENDS

Fig. 1. Effects of suramin on growth of c-Ha-*ras* transformed NIH 3T3 RS485 cells. Cells were plated in 6-well plates (35,000 cells/well) and after 24 hours the medium was changed to contain either 0  $\mu$ M ( $\square$ ), 100  $\mu$ M ( $\blacktriangle$ ), 125  $\mu$ M ( $\bullet$ ), or 150  $\mu$ M ( $\triangle$ ) suramin. Alternatively NIH 3T3 cells were grown without suramin ( $\blacksquare$ ). At the indicated times, cells were stained with crystal violet, quantitated by spectrophotometry at 590 nm, and growth curves obtained. Each data point is the average of 3 determinations  $\pm$  SD. Inset: morphology of RS485 cells grown in the absence (-Suramin) or constant presence of 150  $\mu$ M suramin (+Suramin) until confluent. Cultures were photographed with a phase contrast microscope (Zeiss) equipped with a conventional Nikon camera.

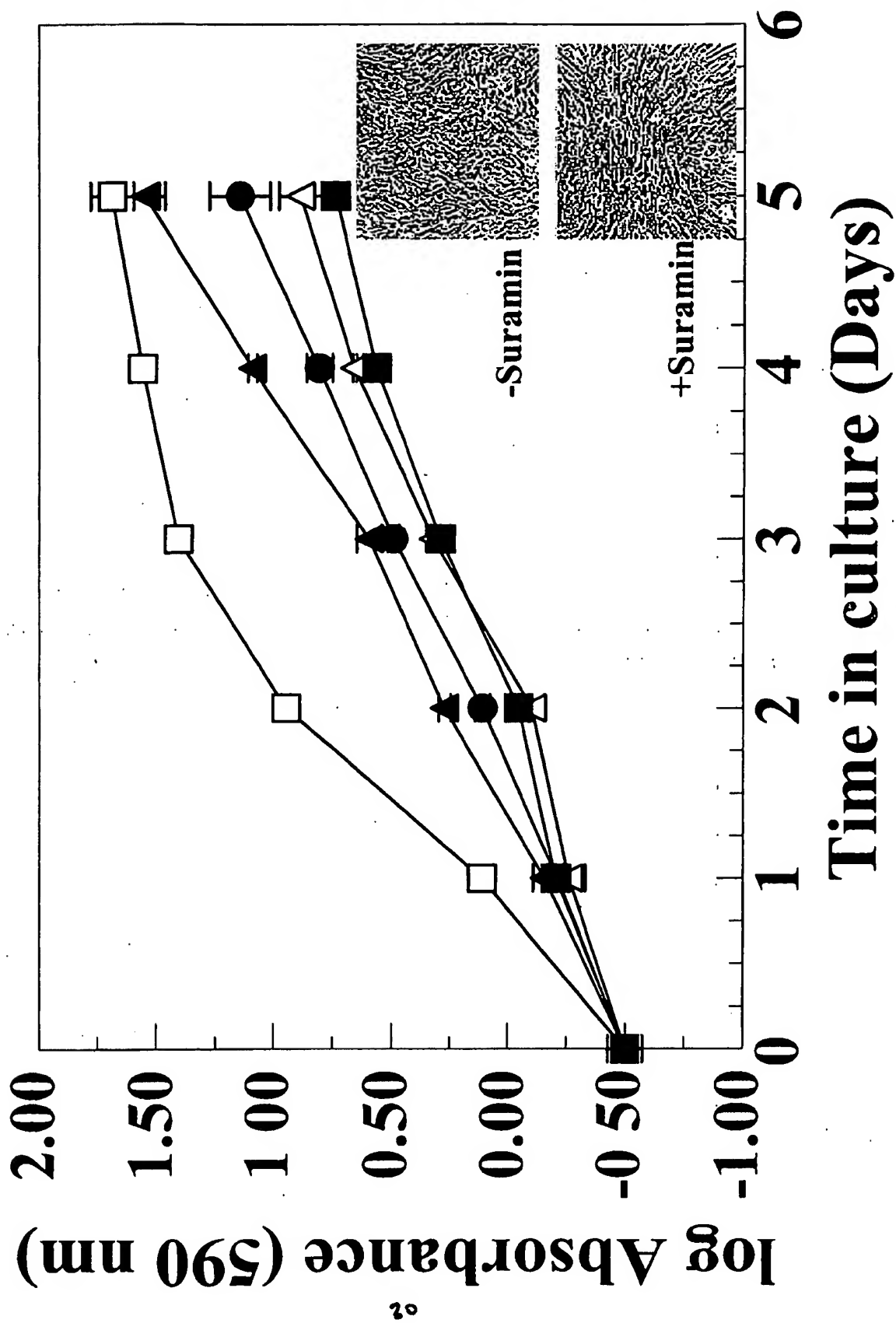
Fig. 2. Effects of BAPN on the growth of RS485 cells and PR4 cells. (A) RS485 cells were plated in 6-well plates (35,000 cells/well) and grown continuously in the absence of additions ( $\bullet$ ), or in the presence of 150  $\mu$ M suramin ( $\blacksquare$ ), 400  $\mu$ M BAPN ( $\triangle$ ), or of both 150  $\mu$ M suramin and 400  $\mu$ M BAPN ( $\circ$ ). (B) PR4 cells were plated in 6-well plates (35,000 cells/well) and grown continuously in the presence of 0 ( $\blacktriangle$ ) or 400  $\mu$ M ( $\bullet$ ) BAPN. Growth curves for both (A) and (B) were generated as described in the legend to Figure 1. Each data point is the average of values from 3 wells  $\pm$  SD.

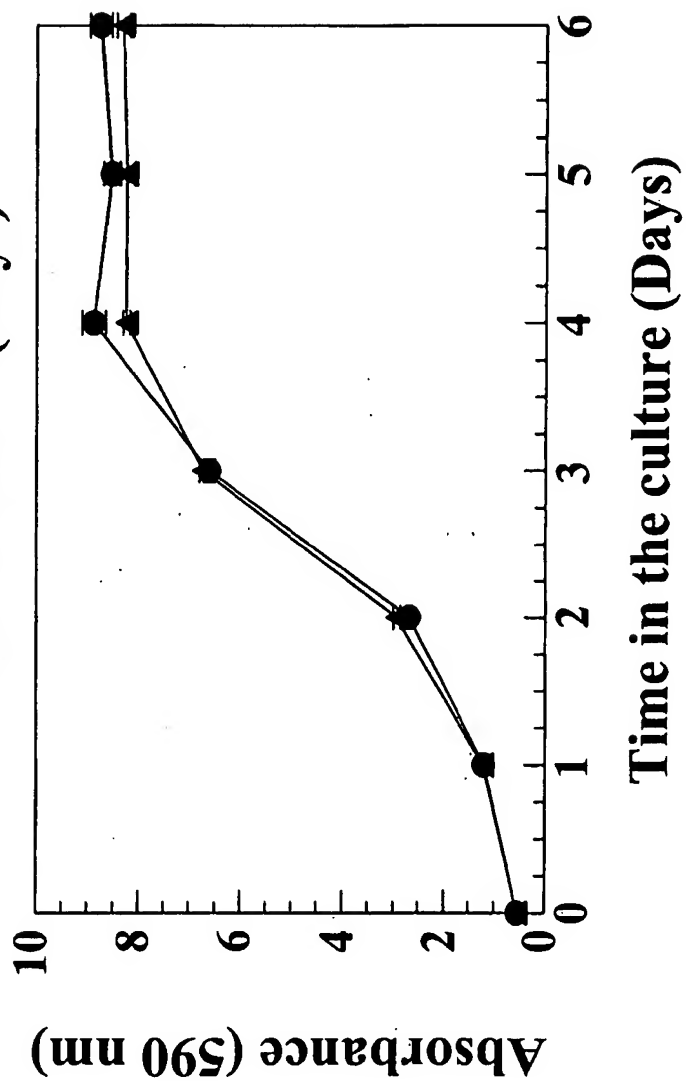
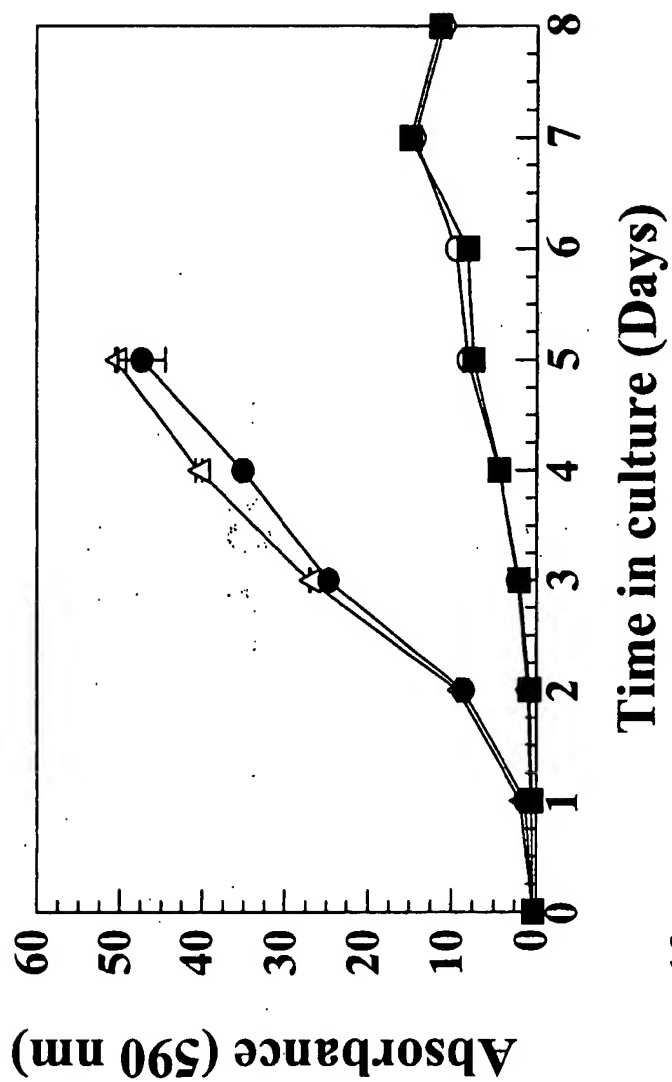
Fig. 3. Effect of suramin on the cell cycle progression of anti-sense lysyl oxidase transfected RS485 cells. Anti-sense lysyl oxidase transfected and empty vector transfected cells were grown in the presence of 0 or 150  $\mu$ M suramin for 24 hours. Cells ( $1.5 - 2 \times 10^6$ ) were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. Data shown are the average difference of the percentage of cells in G1 and S phase with suramin treatment of anti-sense lysyl oxidase transfected cells (L), empty vector transfected cells (V), or non-transfected cells (N), shown for comparison. Nine and six clones from anti-sense lysyl oxidase and empty vector transfected RS485 cells respectively were used for the experiment. (\*,  $p < 0.01$ , t-test assuming equal variances).

Fig. 4. Anti-sense lysyl oxidase reduces suramin-induced increases in normalized lysyl oxidase mRNA levels in RS485 cells. (A) Two representative clones of anti-sense lysyl oxidase transfected (L) and empty vector transfected (V) RS485 cells were grown in the absence (-) or the presence (+) of 150  $\mu$ M suramin. Ten  $\mu$ g of RNA were subjected to Northern blot analysis, for lysyl oxidase mRNA and 18S rRNA levels. (B) Quantification of lysyl oxidase levels normalized to 18S rRNA. Values represent the mean  $\pm$  SD obtained from three scanning densitometry determinations.

Fig. 5. Effects of lysyl oxidase pro-peptide on the growth of RS485 cells. Cells were plated in 24-well plates (7,000 cells/well) pre-coated with 0  $\mu$ g (■), 0.2  $\mu$ g (□), 1  $\mu$ g (▲), 2  $\mu$ g (○), or 4  $\mu$ g (●) recombinant rat lysyl oxidase pro-peptide/well. Growth was assessed as described above in Fig. 1. Each data point is the average of values from 3 wells  $\pm$  SD.

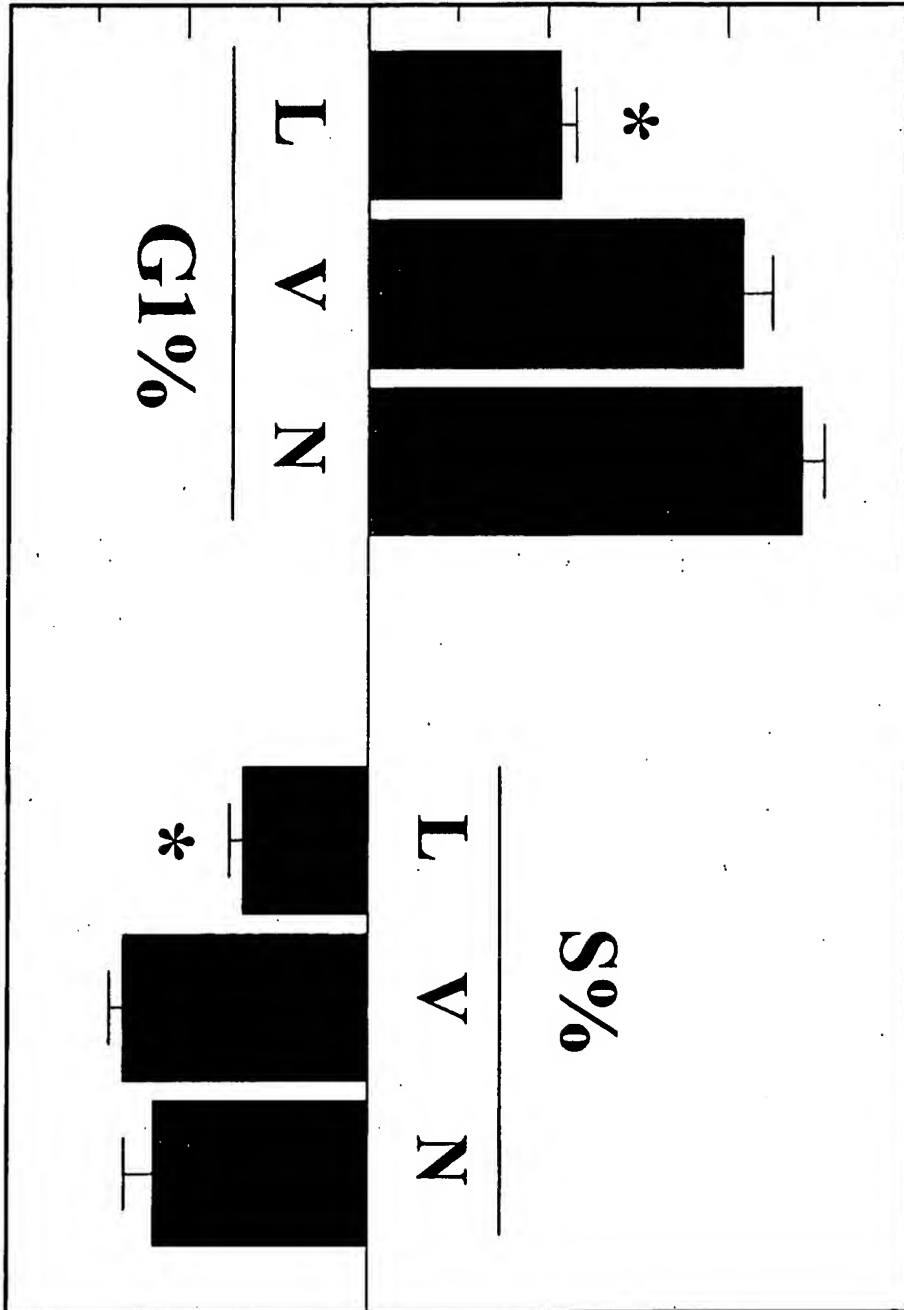
Fig. 6. Lysyl oxidase pro-peptide inhibits growth of *ras*-transformed cells in soft agar. (A) Crystal violet stained colonies of c-Ha-*ras* transformed NIH 3T3 cells (*ras*) or *myc*-transformed M158 cells (*myc*) grown in soft agar in the presence of bovine aorta lysyl oxidase enzyme (+LO), recombinant rat lysyl oxidase pro-peptide (+LO pro-peptide), or vehicle control. (B) Colonies were counted in three independent fields, and values per field  $\pm$  SD expressed as percent of vehicle control.



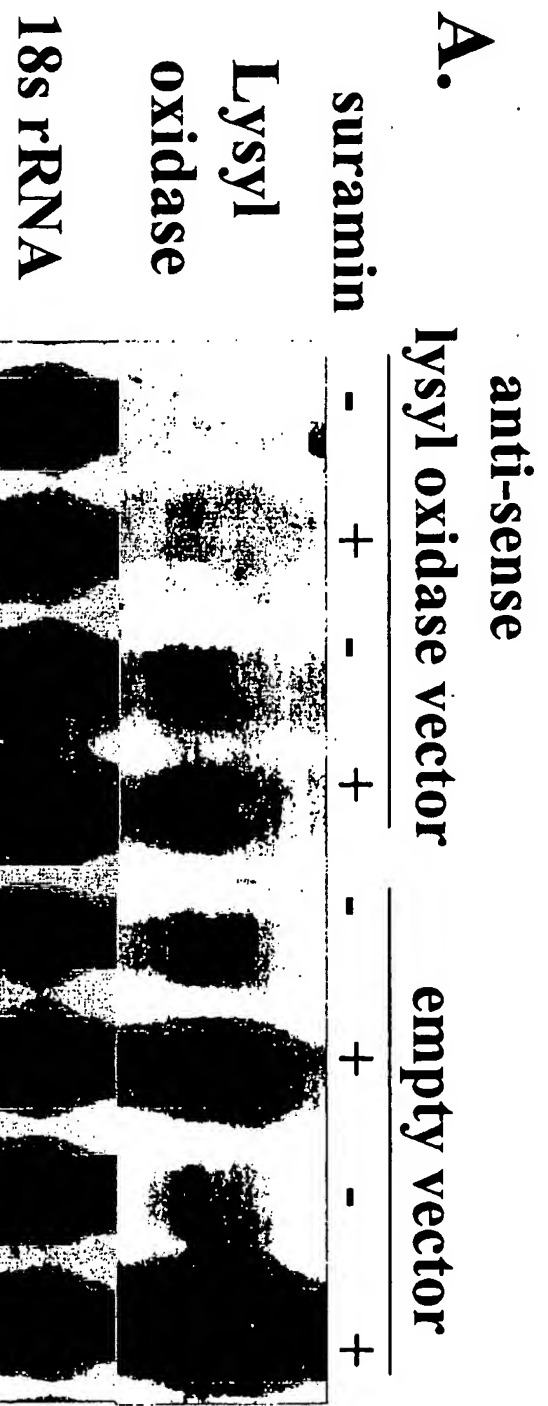


# Average difference

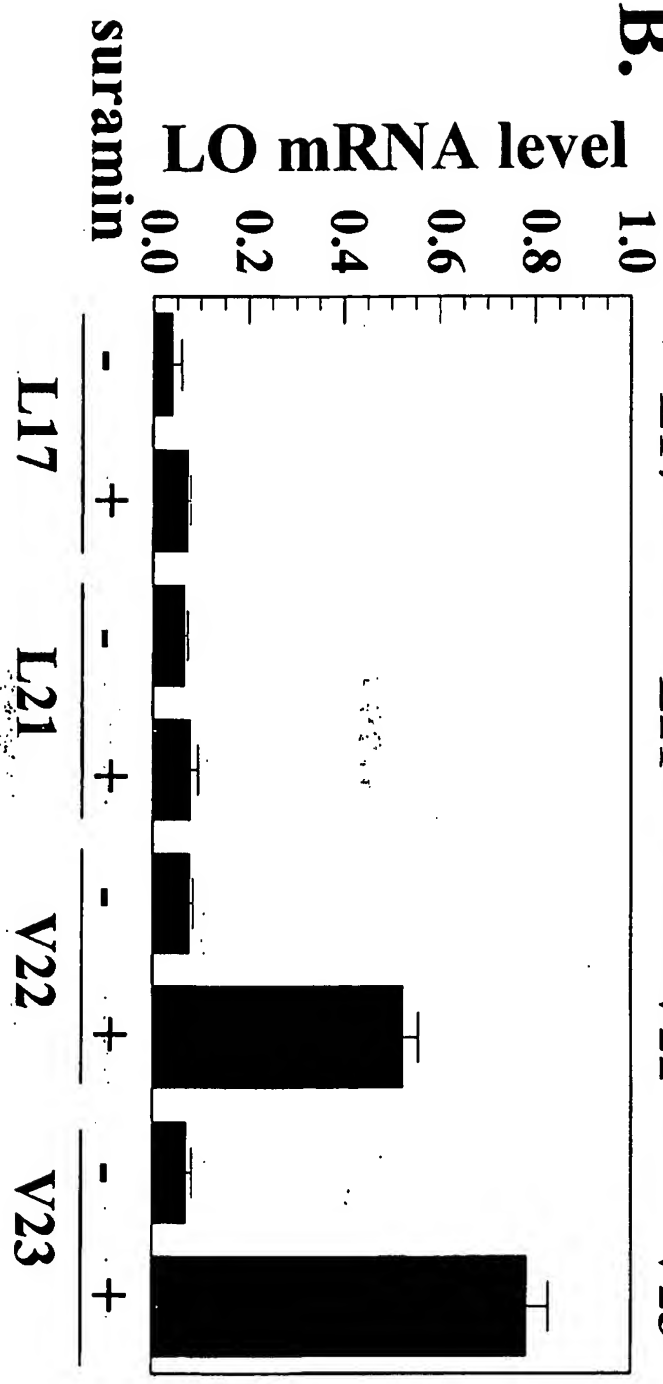
30.0  
20.0  
10.0  
0.0  
-10.0  
-20.0



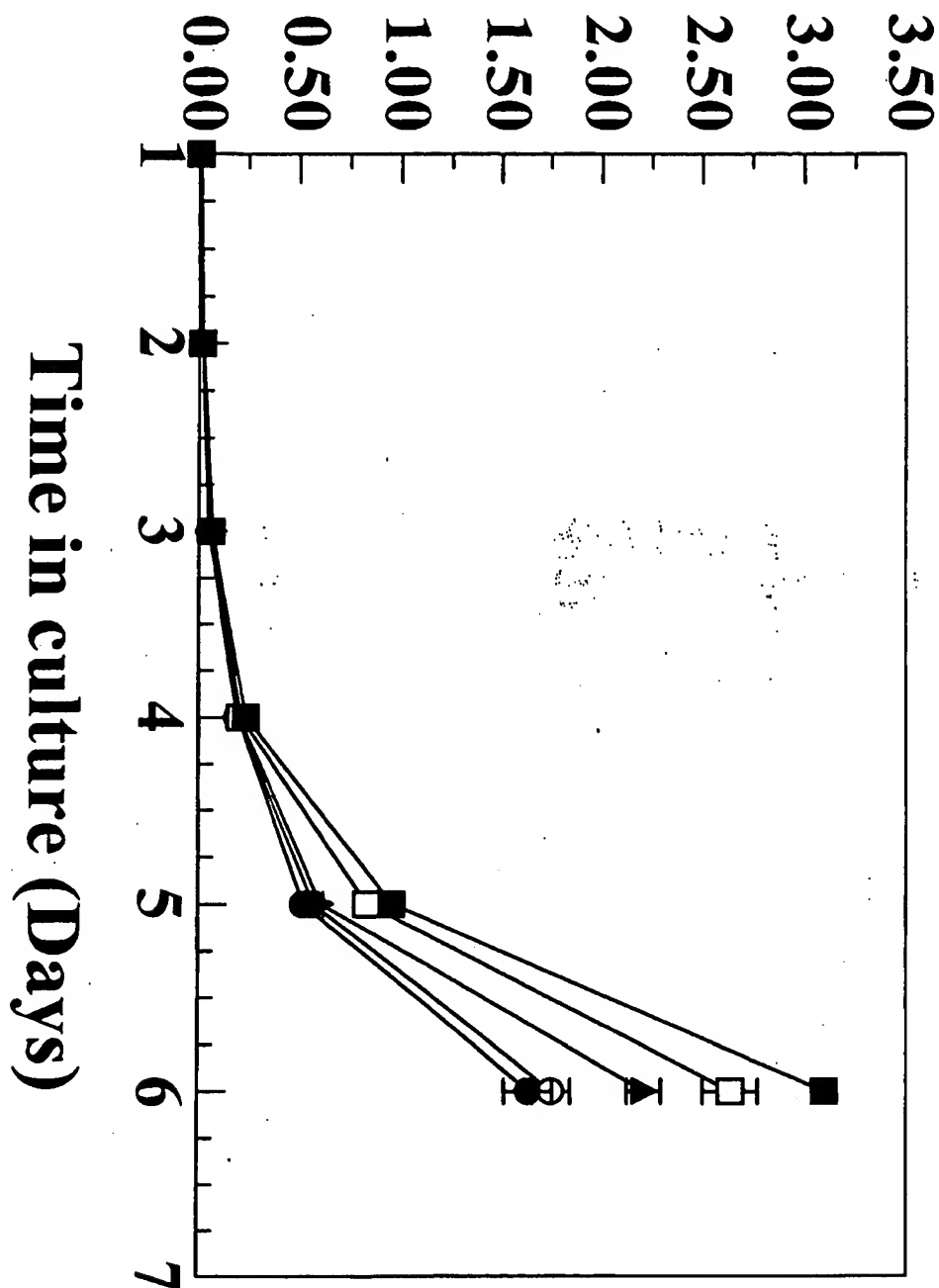
**A.**



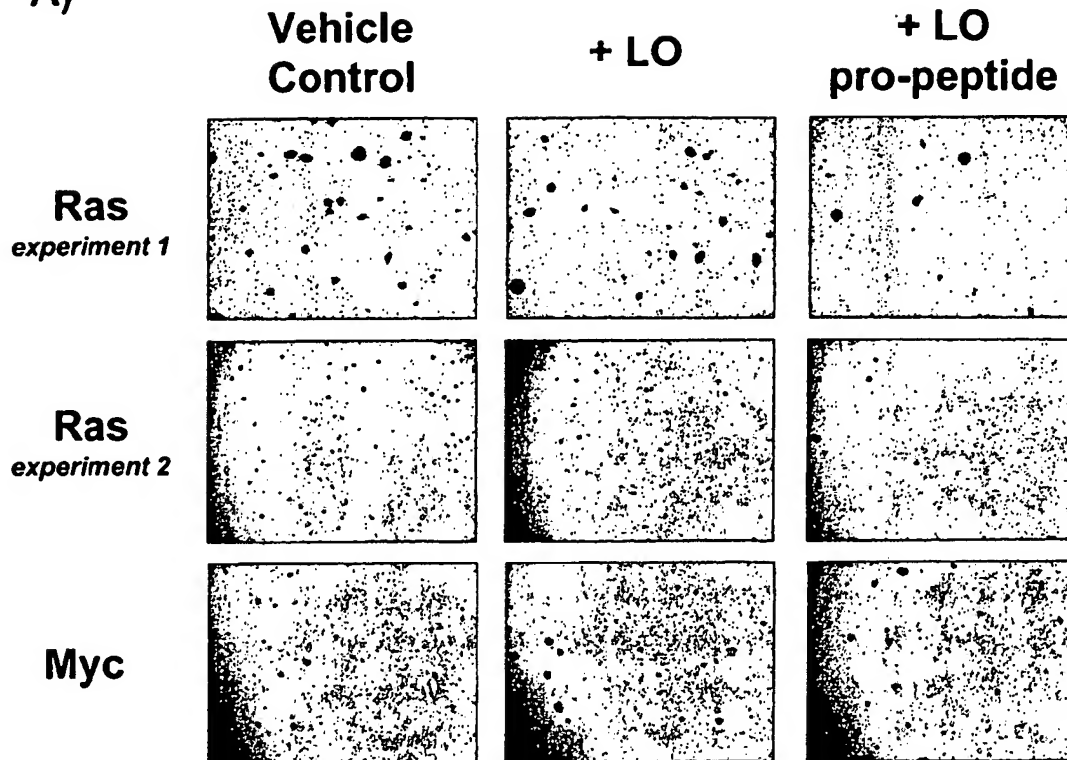
**B.**



# Absorbance (590 nm)



A)



B)

